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Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron

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ABSTRACT

Biofilm formation of *Bacillus cereus* reference strains ATCC 14579 and ATCC 10987 and 21 undomesticated food isolates was studied on polystyrene and stainless steel as contact surfaces. For all strains, the biofilm forming capacity was significantly enhanced when in contact with stainless steel (SS) as a surface as compared to polystyrene (PS). For a selection of strains, the total CFU and spore counts in biofilms were determined and showed a good correlation between CFU counts and total biomass of these biofilms. Sporulation was favoured in the biofilm over the planktonic state. To substantiate whether iron availability could affect *B. cereus* biofilm formation, the free iron availability was varied in BHI by either the addition of $FeCl_3$ or by depletion of iron with the scavenger 2,2-Bipyridine. Addition of iron resulted in increased air–liquid interface biofilm on polystyrene but not on SS for strain ATCC 10987, while the presence of Bipyridine reduced biofilm formation for both materials. Biofilm formation was restored when excess $FeCl_3$ was added in combination with the scavenger. Further validation of the iron effect for all 23 strains in microtiter plate showed that fourteen strains (including ATCC10987) formed a biofilm on PS. For eight of these strains biofilm formation was enhanced in the presence of added iron and for eleven strains it was reduced when free iron was scavenged. Our results show that stainless steel as a contact material provides more favourable conditions for *B. cereus* biofilm formation and maturation compared to polystyrene. This effect could possibly be linked to iron availability as we show that free iron availability affects *B. cereus* biofilm formation.

1. Introduction

Most microorganisms have the capacity to adhere to surfaces and reside in surface-associated, multicellular communities called biofilms. Within a biofilm, cells are held together in a self-produced extracellular matrix that typically consists of extracellular polysaccharides, proteins and sometimes eDNA (Branda et al., 2005). Embedded in the biofilm, cells are protected from harsh environmental conditions including physical stresses, chemicals, and antimicrobial components because the matrix acts as a protective barrier that limits the penetration of disinfectants into the bulk of the biofilm. This can cause a problem for the food industry (Marchand et al., 2012) as equipment surfaces where biofilms develop can become a source of product recontamination (Eneroth et al., 2001; Flint et al., 1997). The lifecycle of a biofilm is a multistep process involving attachment of planktonic cells to a surface, biofilm development and maturation, and eventually disassembly and release of the cells. The biofilm development process is regulated via multiple regulatory pathways that trigger its formation depending on the environmental conditions (Vlamakis et al., 2013). Attachment is known to be influenced by numerous factors such as physicochemical properties of the substratum surface (Flint et al., 2000), surrounding environment, cell surface characteristics (Vanhaecke et al., 1990) and other factors as reviewed in Palmer et al. (2007) and Goulter et al. (2009). However, the importance of surface characteristics seems to vary between reported studies and it has been suggested that this reflects the existence of different mechanisms of adhesion employed by individual microorganisms (Tresse et al., 2007). Besides the substratum surface characteristics and strain diversity, biofilm formation is also influenced by a multitude of other factors including environmental conditions, nutrient availability, presence of specific organic and inorganic molecules in the environment that can act as signals for biofilm formation (Karatan and Watnick, 2009; Petrova and Sauer, 2012). One of such molecules is iron, which is also the main component of stainless steel widely used in industrial environments. Iron has been shown to promote biofilm formation by several microorganisms, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Banin et al., 2005; Lin et al., 2012; Trappetti et al., 2011; Wu and Outten, 2009). On the other hand, iron has also been shown to prevent biofilm formation by *Legionella pneumophila* (Hindré et al., 2008) and *Streptococcus mutans* (Berlutti et al., 2004).

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Bacillus cereus is a food spoilage and pathogenic sporeformer. Different studies report biofilm formation by *B. cereus* on abiotic surfaces including stainless steel, polystyrene and glass (Auger et al., 2006; Houry et al., 2010; Karunakaran and Biggs, 2011; Lindsay et al., 2006; Wijman et al., 2007) but a direct comparison between different substrata has not been performed. Within the established biofilms, *B. cereus* is able to form spores (Faille et al., 2014; Lindsay et al., 2006; Wijman et al., 2007) which may lead to product contamination upon release. The objective of this study was to assess the biofilm forming capacity of 21 *B. cereus* food isolates and two reference strains, ATCC 14579 and ATCC 10987 on stainless steel and polystyrene and to evaluate the influence of iron. For a selection of strains, these biofilms were characterised in more detail by correlating the total biomass with cell counts. Spore numbers in the biofilms were also determined. The data provided in this study show a large diversity in biofilm forming capacity between the different *B. cereus* isolates and show a preference for stainless steel as contact surface over polystyrene. We also show that the free iron availability may enhance biofilm formation of several *B. cereus* strains.

2. Materials and methods

2.1. Strains and culturing conditions

Twenty-one *Bacillus cereus* strains previously isolated from food products and supplied by food manufacturers, were used in this study and compared for biofilm formation with the *B. cereus* reference strains ATCC 10987 and ATCC 14579 (Table 1). The strains were identified as *Bacillus cereus* based on performance on *Bacillus cereus* selective media and ribosomal RNA sequencing. Strains were streaked on BHI (Brain Heart Infusion, Becton Dickinson, France) agar plates from stocks stored at −80 °C and incubated for 24 h at 30 °C to obtain single colonies. A single colony was used to inoculate BHI broth and incubated overnight (18 h) at 30 °C without shaking. Preliminary tests using BHI, TSB, LB and a chemically defined minimal medium (Mols et al., 2007) showed that BHI was the optimum medium supporting biofilm formation (data not shown) and was therefore selected for subsequent experiments.

Table 1
B. cereus strain codings used in this study and their source of isolation.

B. cereus strain designation used in this study	Strain designation according to NIZO culture collection	Source of isolation
BC1	4077	Chilled dessert
BC2	4078	Unknown
BC3	4079	Canned chocolate beverage
BC4	4080	Dried onion
BC5	4081	Provolone sauce
BC6	4082	Asparagus ham sauce
BC7	4083	Tortelini con fungi
BC8	4084	Indian rice dish
BC9	4085	Asparagus soup
BC10	4086	Boiled rice
BC11	4087	Pea soup
BC12	4088	Dressing
BC13	4116	White sauce
BC14	4117	Pasteurised milk
BC15	4118	Ice cream
BC16	4120	Water
BC17	4147	Quiche
BC18	4149	Cooked ham
BC19	4153	Smoothie
BC20	4155	Beef salad
BC21	4158	Gas packaged, cooked and spiced potatoes
ATCC 14579	Reference strain	Air from a cow shed
ATCC 10987	Reference strain	Spoiled cheese

2.2. Biofilm formation and quantification

Biofilm formation by selected *B. cereus* isolates was tested in 12-well plates (Cellstar, suspension culture plate, Greiner bio-one, Germany) which were half filled with 3 ml broth and inoculated with 1.5% overnight-grown cultures. Biofilms were allowed to develop on stainless steel (SS, AISI 304, surface finish 2B) or polystyrene (PS) coupons (22 mm wide, 18 mm height) placed vertically in the wells. Coupons were washed and sterilised prior to use as described by Castelijn et al. (2013). Around half (2 cm^2 on each side) of the total surface area of the coupons was submerged into liquid medium. Coupons were only used once and were discarded after the experiment. Plates were wrapped with parafilm to prevent evaporation during incubation for 24 and 48 h at 30 °C. Biofilms on the coupons were quantified using the crystal violet (CV) assay for total biofilm formed and plate counting to determine the number of culturable cells in the biofilm.

Total biofilm was visualised and quantified by staining with CV as described previously (Castelijn et al., 2013) with the following modifications: coupons with biofilms were gently washed by dipping 2 times in de-mineralised (demi) water and left in 0.1% CV (MERCK) for 30 min to stain. After staining the coupons were washed again 3 times in demi water and subsequently de-stained in 4 ml 70% ethanol for 45 min. Two hundred μl of this ethanol with dissolved CV was transferred to a 96-well plate to measure the OD at 595 nm. The obtained OD values served as quantitative measures of the total biofilm biomass.

In order to determine culturable cells in the biofilm, coupons were washed by dipping 3 times in phosphate-buffered-saline (PBS) and placed in 50 ml tubes filled with 3 ml PBS and 0.5 g glass beads (D = 100 μm, SIGMA). Tubes were vortexed at maximum speed (VortexGenie2, SI, USA) for 1 min to detach the cells from the coupon and to obtain individual cells in the sample. Preliminary experiments showed that this methodology effectively separated cells from each other and from the substratum and did not affect cell viability. Serial dilutions were made and spread plated on BHI-agar plates and colony forming units (CFU) were counted after 24 h incubation at 30 °C. For spore counts, the suspended biofilm was heated at 80 °C for 10 min to inactivate all vegetative cells prior to plating. Initial attachment of cells to the SS coupons was determined after 2 h incubation time following the same approach as described above for the biofilm CFU counts.

For Scanning Electron Microscopy (SEM) SS and PS coupons with biofilm were washed 3 times in PBS and the procedure described previously for preparation of the samples was followed (Castelijn et al., 2012).

2.3. The role of iron in biofilm formation

To assess the role of iron, biofilm formation was measured in the following 4 conditions: BHI as a control; BHI supplemented with iron ($FeCl_3$) to a final concentration of 250 μM added $FeCl_3$ (MERCK); BHI supplemented with 450 μM 2,2-Bipyridine (MERCK) (BHI + Bip); and BHI supplemented with both $FeCl_3$ and 2,2 Bipyridine (BHI + Bip + $FeCl_3$). The concentrations used allowed growth of most of the strains and were selected based on pilot studies showing a significant effect on biofilm formation. The filter sterilised (25 and 45 mM for $FeCl_3$ and 2,2 Bipyridine respectively) stocks of these components were added to BHI broth just before inoculation. Biofilms formed on the coupons in 12-wells were measured for the reference strain ATCC 10987.

Screening of all *B. cereus* strains was performed in a microtiter plate assay (Djordjevic et al., 2002) in 96-wells plates filled with 200 μl BHI inoculated with 1.5% overnight culture of the respective strains with or without $FeCl_3$ and/or Bipyridine supplementation. The amount of biofilm formed was measured after 24 h with the CV assay, with the difference that biofilms analysed were formed on the walls of the wells instead of coupons. Washing, staining and de-staining steps were performed using 250 μl of appropriate solutions and the OD measurement at 595 nm was performed in the same 96-well plate. The